Mechanically-Induced Transition of Human Induced Pluripotent Stem Cells

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Abstract

Human induced pluripotent stem cells (hiPSCs) grown in multiple culture media were altered after being subjected to a stretching protocol. hiPSC maintenance media and Gray's media were used during the 6 day stretching protocol of 0.1 Hz with 5% circular elongation. While the final state of their development remains a mystery, qPCR using pluripotent, endoderm, and lung markers confirmed that the cells had transitioned from hiPSCs but had not been differentiated into one of these other cell lines. Further investigation must be performed to determine the cells' new identity. Variables that could be modified in the future include cultureware coating, media used, and stretching protocols.

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Introduction

A variety of different diseases can affect lung health, many of which cause irreversible lung damage. Some of these include chronic obstructive pulmonary disease (COPD), emphysema, and cystic fibrosis. In 2011, chronic lower respiratory diseases were the third leading cause of death in the USA.[1] These diseases are usually treated with drugs and other common medical practices. Unfortunately, these drugs and practices are not very effective and cannot reverse damage done by the disease. Transplantation is a very attractive treatment option because it offers an entirely new organ, and it is also the only permanent option for patients with diseases that cause irreversible lung damage.[2] Unfortunately, there are not enough donor lungs to fulfill this demand, and the few lung transplantation performed often result in chronic rejection which can lead to obliterative bronchiolitis, the leading long-term cause of mortality after lung transplantation.[3] In order to prevent this, bioengineered tissues using host-derived cells are being developed.[4] In order to do this, somatic cells are acquired from the host, and 'reprogrammed' into human induced pluripotent stem cells (hiPSCs) using a variety of transcription factors such as Oct3 and Sox2.[5] These hiPSCs are undifferentiated cells that can develop into different types of cells as a result of environmental conditions including substrate stiffness, chemical factors, and mechanical stimulation. To bioengineer lungs, these hiPSCs can be differentiated into endoderm progenitor cells (EPCs), which can further develop into cell types unique to the lungs.[6]

These cells can be grown via traditional culture plate methods or injected into a matrix scaffold. Man-made matrices lack the intricacy and complexity present in a real lung, which can be decellularized to its extracellular matrix (ECM) components. This removes all genetic traces of the lung's original host while leaving its structure and basic chemical properties, making it an attractive option for transplantation free of rejection.[4]

In addition to substrate differences, various cell culture media can also have a large effect on how hiPSCs behave and differentiate. hiPSC maintenance media can be used to create a homeostatic environment for the cells and help them maintain pluripotency. In 1996, Gray et al. reported that a media with increased retinoic acid concentration had a significant effect on promoting mucociliary differentiation of lung epithelial cells.[7] While this experiment was performed on lung epithelial cells, Gray's media could have significant effects on hiPSCs, too.

Mechanical factors can also influence stem cell differentiation. Teramura et al. discovered that cells cyclically stretched to a 15% surface area increase for 12 hours at 0.2 Hz had reduced expression of Sox2 and other hiPSC factors.[8] By modifying the rate and intensity of the stretching protocol, various *in vivo* situations can be modeled. For example, embryonic and fetal lung development begins around week 4 of pregnancy, and breathing rates begin at week ten.[9, 10] The rate increases from that point forward, and that rate could be used *in vitro* to mimic conditions at that stage in lung development.

A combination of all of these factors could result in new discoveries to induce differentiation of hiPSCs. By experimenting with new and existing media, culture matrices, and stretching protocols, cell culture regimens could be tailored to mimic *in vivo* conditions and promote cellular differentiation into various cell lineages. This knowledge and these cells could one day be used to reseed existing lung matrices. These personalized matrices will facilitate the lung transplantation process for the millions of people suffering from lung diseases.

The purpose of this study was to investigate how a combination of the mechanical, chemical, and structural factors discussed above can be tailored to direct the differentiation of hiPSCs into EPCs, which may one day be further differentiated in order to bioengineer functional lungs.

Materials and Methods

There were four cellular conditions for this experiment. Cells were cultured in either hiPSC maintenance media or Gray's media, and within each of these conditions there were stretched and unstretched cells. The cellular seeding, feeding, and harvesting processes are described here and a timeline is shown in Figure 1.

hiPSC Culturing

hiPSCs were originally derived from human skin fibroblasts by the Stem Cell Institute at the University of Minnesota. The experimental cells were begun at passage 77. Controls were obtained from the same cell line at passage 72 and are referred to as Control hiPS cells.

Cell Culture Media

There were two cell culture media used in this experiment. The control media was a human embryonic stem (hES) cell media that was described in Park et al.[11] From here on, it is referred to as hiPSC media. The other media was described in by Gray et al. and consists of 1:1 Dulbecco's modified Eagle's medium (DMEM) and bronchial epithelial growth medium (BEGM).[7] The BEGM-supplied retinoic acid (RA) was not added to the media, but supplemental RA was dissolved in DMSO and added to bring the final media RA concentration to 50 nM. This media is now referred to as Gray's media.

PLM Synthesis

Cells were grown on untreated BioFlex® culture plates from Flexcell International Corporation. These were initially plated with pig lung matrix (PLM) solutions. To create these

solutions, the decellularization protocol from Price et al. was modified for application to porcine lungs.[4] Next, the distal regions of these decellularized lungs were removed and lyophilized. ≥2500 U of pepsin and 1 mL of 0.01 M HCl were added per 10 mg of lyophilized tissue, and this was stirred at room temperature. 4.5 mL of the PLM solution was diluted with 19.5 mL of sterile PBS and then evenly distributed among 2 plates (12 total wells). These plates incubated at 37°C for 24 hours before being seeded with cells.

Cellular Plating

24 hours after being coated with PLM, irradiated (3000 cGy) CF-1 mouse embryonic fibroblasts (MEFs) feeders were added to the plates (approximately 500,000 cells/well). 24 hours later, on day 0, hiPSCs were plated in 3 mL hiPS media per well. On day 1, 2 mL hiPSC media were added to each well of hiPSCs, and 2 mL Gray's media were added to the Gray's cells. Cells were then fed with the 6 mL per well of the appropriate media on days 2, 4, and 6.

Stretching

On day 1, a sinusoidal stretching protocol of 0.1 Hz with a maximum 5% circular elongation was begun. A Flexcell® FX-4000™ Tension System was used to perform the stretching, and this protocol was maintained constantly except for when cells were removed to be fed or to take pictures. On day 4, the loading posts were switched to custom posts that were 1/16 inches taller to accommodate the plastic deformation of the cell culture surface over the previous days of stretching. The stretching regimen was chosen because there is no fetal breathing at week 4 (when the lungs first begin to develop from hES cells), and this is the slowest stretch rate we

could perform with the Flexcell® FX-4000™.[9] In addition, 5% maximum elongation successfully strained the cells without subjecting enough stress to kill them.

Removal and Analysis

On day 6, the cells were coated with trypsin, incubated for 5 minutes, and then removed from the plates. Then the cells were lysed using 1 mL of Life Technologies TRIzol® per test condition. Then an RNA isolation was performed using a PureLink® RNA Mini Kit from Life Technologies. A cDNA library was created using an Invitrogen Superscript III kit. Then a qPCR was performed with the five Life Technologies TaqMan® marker primers. GAPDH was used as a standard reference, Oct-4 and SOX2 were used as stem cell markers, and SOX17 and TTFI were used as endoderm and early lung progenitor markers, respectively.

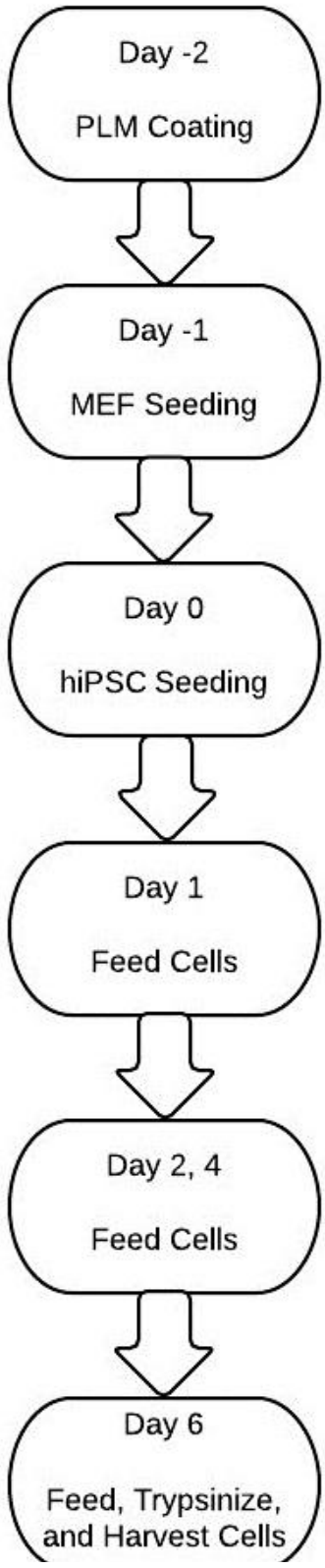

Figure 1 is a flow diagram showing the cellular seeding and harvesting timeline.

Results

Photographs of the cells during the experiment can be seen in Figure 2.

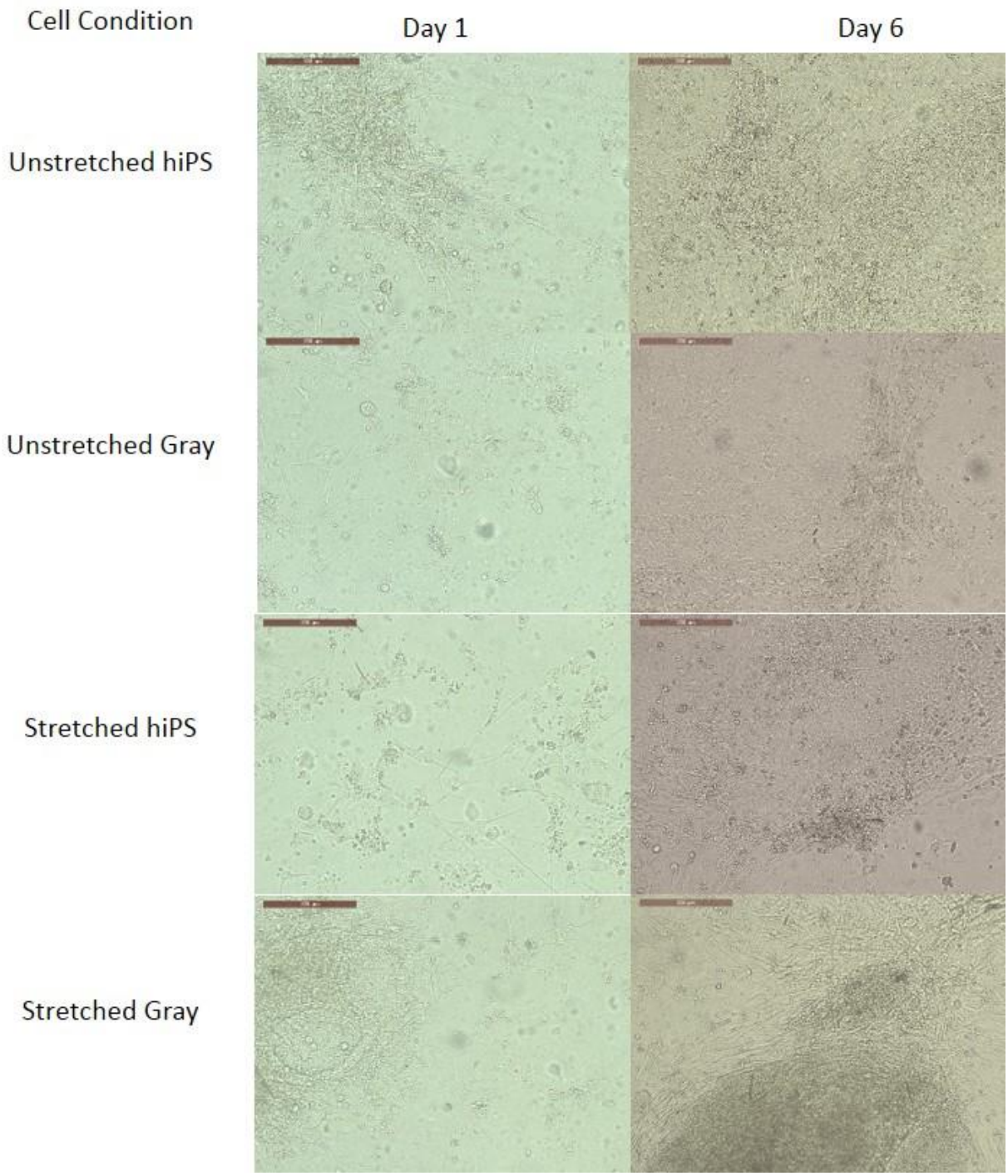

Figure 2 shows photographs of some of the cells used in the experiments. The columns are cells from days 1 and 6, while the rows are for each unique media and stretching combination.

After the RNA isolation, the following RNA metrics recorded in Figure 3.

Table 1 - RNA Data					
Sample	Control hiPS (p72)	Unstretched hiPS	Unstretched Gray	Stretched hiPS	Stretched Gray
Concentration (µg/mL)	36.5	1123	1196	767	1202
A260/A280	3.167	2.028	2.046	2.089	2.070

Figure 3 shows the concentrations and A260/A280 values for the RNA extracted from all cells in the study.

Finally, a qPCR was performed. GAPDH was the standard, and the markers used were Oct-4, SOX2, SOX17, and TTF1. Oct-4 and SOX2 are both stem cell markers, SOX17 is an endoderm marker, and TTF1 is an early lung progenitor marker. TTF1 was undetected, and SOX17 was amplified at such late counts that this data was unreliable. Relative quantities (RQ) of the other expression markers are shown in Figure 4.

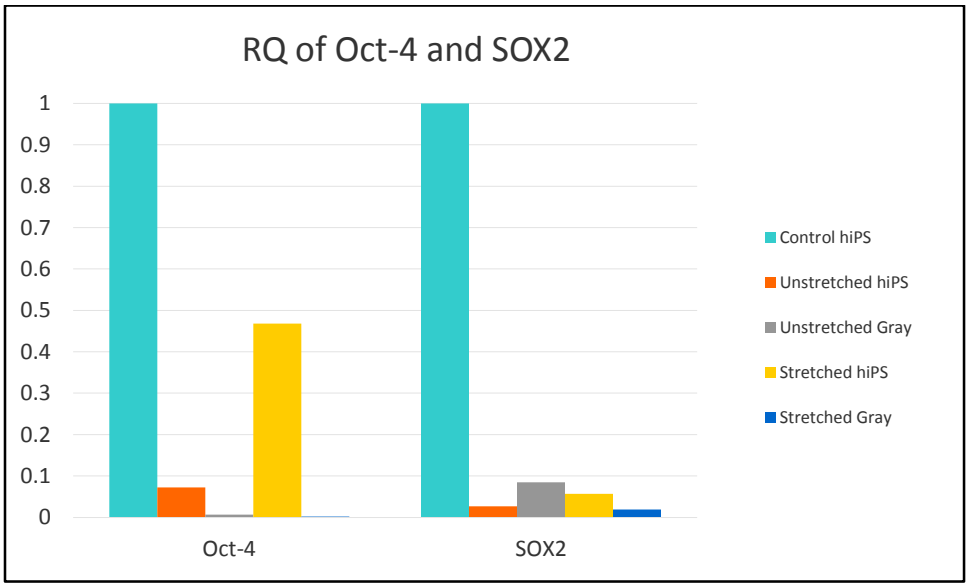

Figure 4 shows the relative quantities of Oct-4 and SOX2 after performing the qPCR on all cells. The Control hiPS cells were the controls, and GAPDH was used as a RQ standard

Discussion

As shown above, a transition of the hiPSCs did occur because hiPSC markers Oct-4 and SOX2 were not amplified by the qPCR. However, there was a substantial amount of Oct-4 present in the stretched hiPSCs, indicating that the stretching protocol may have helped the cells maintain some aspects of pluripotency in the presence of the PLM coating. Other than that, it appears that this stretching protocol had little effect on the cells' transition. The experiment also shows that the cells did not become EPCs or lung progenitors, as markers SOX17 and TTF1 were also not present. This leaves a number of different options for the cells' identity. hiPSCs have been successfully differentiated into various neural, cardiac, and hematopoietic progenitor cells, but they have the ability to generate all cell types.[12] Further investigation must be done to determine if the cells were differentiated into one of these cell lineages. In order to do this, qPCR would need to be repeated with a variety of markers to explore the known cellular spectrum to see what types of cells have been generated.

Figure 2 shows photographs of the cells at day 1 and 6. While no cell count was performed after the experiment, it is apparent that there is a higher concentration of cells in the day 6 pictures, demonstrating that the cells did indeed proliferate over the course of the experiment. In addition, the MEFS are visible as longer cells underneath the rounder hiPSCs, indicating that these were still present to provide nourishment to the hiPSCs throughout the experiment. An additional comment about the pictures is that the reason for inconsistent cellular coating of the surface is likely due to protein aggregates from the PLM coating. Some of the open sections of the stretched images could be due to release of PLM and cells from the cell culture surface during stretching.

The table in Figure 3 shows that the hiPS Control RNA concentration was the lowest of the samples, and that the Stretched hiPS had a lower concentrations, indicating that these were the least prolific cells studied. The A260/A280 values, a metric for nucleic acid quality, are all near 2 for the tested cells, which indicates a very pure sample.[13] The controls had a value of 3.167, but this is still in an acceptable range for an RNA samples. However, it is important to note that these control cells, while obtained from the same cell line as the tested cells, were not harvested at the same passage. This means that some biochemical changes may have occurred in the tested cells before the experiment occurred. However, these cells were grown with hiPSC maintenance procedures, so they should have still retained their pluripotency until the experiment began.

One potential pitfall in this project is the use of decellularized pig lung tissues versus human ones. Porcine samples are much easier to obtain and were therefore the ideal choice for this experiment. Furthermore, as the tissues were decellularized to the ECM, there should be very little difference between the biochemical properties of porcine and human samples. A larger concern is the method of coating tissue culture plates with the PLM. In order to better mimic *in vivo* lung development, the hiPSCs should be differentiated within a 3-D gel as opposed to a 2-D surface. However, complications involved with developing, characterizing, and implementing this gel were out of the scope of this project. Even within the 2-D plate, it was difficult to ensure an even and successful PLM coating. Fluorescent microscopy was attempted to quantify the PLM binding on the plates, but autofluorescence of the plates made this difficult. Acid etching with nitric acid was also attempted to improve non-specific binding of PLM components to the plates, but it was not found to be quantifiably better than the methods used in this experiment. The PLM coating effectiveness was finally determined via simple visual inspection. The original

untreated plates were hydrophobic and as a result water droplets and cell culture media beaded up and didn't wet the surface. After the PLM coating used in this paper was performed, these liquids were able to wet the surface, indicating that a chemical change on the surface had occurred. This change was assumed to be a result of the presence of PLM matrix adhered to the plates.

Another future source of experimentation is within the cell culture media. Hundreds, if not thousands, of media recipes exist for different purposes, and this experiment only tested two of them. For example, STEMdiff™ media has been successfully used to derive definitive endoderm from human embryonic stem cells (hESCs).[14] Unfortunately, this media's components are very well guarded, so the only option to use it is to either buy it or develop it in-house. Legartová et al. also highlight the importance of RA in the EPC-like differentiation of mouse embryonic stem cells (mESCs), similar to the Gray's media.[14] In addition to this, there are infinite combinations of chemical factors to induce differentiation, as well as the options of feeders. Normally, hiPSCs are grown on CF-1 MEF feeder cells (as performed here), but there are also options for feeder-free conditions including DSR (high-glucose DMEM-containing knockout serum replacer, glutamine, nonessential amino acids, and β-mercaptoethanol) with knockout serum and ANK (recombinant activin A, human recombinant keratinocyte growth factor, and nicotinamide).[15] Further experimentation with all of these variables is necessary, and ideally a feeder-free option could be used to enhance the consistency and simplicity of the procedure.

Stretching protocol is another variable that should be investigated further. The stretching protocol performed here was limited by the capabilities of the setup, as well as the hardiness of the cells. Teramura et al. performed shorter experiments at higher strain and frequency (12 hours,

15%, 0.2 Hz) and found that reduces expression of the pluripotency markers Nanog, POU5f1, and Sox2.[8] However, Saha et al. also found that 10% average strain at 0.17 Hz over 14 days, combined with conditioned CM/F+ media inhibited hESC differentiation.[16] Both of these experiments used BioFlex® culture plates, but those used in the Teramura paper were coated with collagen I (without feeder cells) and those used in the Saha paper were coated with BD Matrigel™, a feeder-free coating.[8, 16] The combination of these two papers shows that cyclic loading has the potential to have wide-ranging effects on cell differentiation, and it must be properly tuned to get the desired result. After comparing these protocols, it appears that a shorter duration of faster stretching may be necessary to promote EPC differentiation and minimize stem cell expression as shown in the Teramura paper.

In addition, general substrate stiffness also plays a role on hiPSC behavior, so in the future different culture material could be used to control this parameter.[17] Incubator temperature could also have an effect on hiPSC development and differentiation, and this is currently being studied indirectly by human embryo development in the US.[18] Finally, different ambient air oxygen and carbon dioxide concentrations could be varied. Kirkegaard et al. found that culture in 20% oxygen reduces developmental rates of human embryos, so that may be an important variable for future, longer-term differentiations.[19]

Conclusions

hiPSCs grown in multiple culture media were altered after being subjected to a stretching protocol. While the final state of their development remains a mystery, they were no longer hiPSCs and did not differentiate into EPCs. Further investigation must be performed to ascertain their true identity. Media conditions, cultureware coating, and stretching protocols should be modified in the future to successfully differentiate hiPSCs to EPCs.

Bibliography

1. Hoyert, D., & Xu, J. (2012). Deaths: Preliminary data for 2011. *National Vital Statistics Reports, 61*(6)

2. Kreider, M., & Kotloff, R. (2008). Selection of candidates for lung transplantation. *Proceedings of the American Thoracic Society, 6*

3. Reichenspurner, H., Girgis, R., Robbins, R., Conte, J., Nair, R., Valentine, V., et al. (1995). Obliterative bronchiolitis after lung and heart-lung transplantation. *The Annals of Thoracic Surgery, 60*(6)

4. Price, A., England, K., Matson, A., Blazar, B., & Panoskaltsis-Mortari, A. (2010). Development of a decellularized lung bioreactor system for bioengineering the lung: The matrix reloaded. *Tissue Engineering, 16*(8), 2581.

5. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell, 131*(5)

6. Franks, T., Colby, T., Travis, W., Tuder, R., Reynolds, H., Brody, A., et al. (2008). Resident cellular components of the human lung: Current knowledge and goals for research on cell phenotyping and function. *Proceedings of the American Thoracic Society, 5*

7. Gray, T., Guzman, K., Davis, C., Abdullah, L., & Nettesheim, P. (1996). Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *American Journal of Respiratory Cell and Molecular Biology, 14*(1)

8. Teramura, T., Takehara, T., Onodera, Y., Nakagawa, K., Hamanishi, C., & Fukuda, K. (2012). Mechanical stimulation of cyclic tensile strain induces reduction of pluripotent related gene expressions via activation of Rho/ROCK and subsequent decreasing of AKT phosphorylation in human induced pluripotent stem cells. *Biochemical and Biophysical Research Communications, 417*(2)

9. Schöni-Affolter, F., Dubuis-Grieder, C. & Strauch, E. (2006). *Human Embryology: 18.1 phases of lung development.*, 2014, from http://www.embryology.ch

10. Govindana, R., Wilsona, J., Murphy, P., Russelc, W., & Lowery, C. (2007). Scaling analysis of paces of fetal breathing, gross-body and extremity movements. *National Institutes of Health Public Access Manuscripts, 386*(1)

11. Park, I., Lerou, P., Zhao, R., Huo, H., & Daley, G. (2008). Generation of human-induced pluripotent stem cells. *Nature Protocols, 3*(7)

12. Amabile, G., & Meissner, A. (2009). Induced pluripotent stem cells: Current progress and potential for regenerative medicine. *Trends in Molecular Medicine, 15*(2)

13. Gallagher, S., & Desjardins, P. (2008). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy, supplement 52, A.4K.1. *Current Protocols in Protein Science*

14. Legartová, S., Kozubek, S., Franek, M., Zdráhal, Z., Lochmanová, G., Martinet, N., et al. (2013). Cell differentiation along multiple pathways accompanied by changes in histone acetylation status. *Biochemistry and Cell Biology, 92*(2)

15. King, C., Beattie, G., Lopez, A., & Hayek, A. (2008). Generation of definitive endoderm from human embryonic stem cells cultured in feeder layer-free conditions. *Regenerative Medicine, 3*(2)

16. Saha, S., Ji, L., de Pablo, J., & Palacek, S. (2006). Inhibition of human embryonic stem cell differentiation by mechanical strain. *Journal of Cellular Physiology, 206*(1)

17. Earls, J., Jin, S., & Ye, K. (2013). Mechanobiology of human pluripotent stem cells. *Tissue Engineering, 19*(5)

18. National Institutes of Health, Clinical Trials, & Fawzy, M. (2013). *Incubator temperature for culturing human embryos.*, 2014, from http://clinicaltrials.gov/ct2/show/NCT01706900

19. Kirkegaard, K., Hindkjaer, J., & Ingerslev, H. (2013). Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertility and Sterility, 99*(3)